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Award Number: W81XWH-05-1-0130

TITLE: Reg IV. A Candidate Marker of Hormone Refractory Metastatic Prostate Cancer

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REPORT DATE: January 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-01-2008	Final	1 JAN 2005 - 31 DEC 2007

4. TITLE AND SUBTITLE

Reg IV. A Candidate Marker of Hormone Refractory Metastatic Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0130

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California
Los Angeles, CA 90095

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In our previous in situ hybridization studies of prostate tissue arrays, RegIV was shown to be strongly expressed by a majority of metastatic hormone refractory tumors (HRPC) and weaker RegIV expression was found in a subset of primary tumors, but not expressed by normal tissue. Our goal is to better understand the role of RegIV in prostate cancer progression and to determine its possible use as a diagnostic marker for early and metastatic prostate cancers. We have developed and affinity purified a polyclonal anti-RegIV that has successfully been tested by immunohistochemistry on positive and negative controls. In vitro studies show cells overexpressing RegIV have a growth advantage, but not in vivo. However, Reg IV knockdown experiment showed that Reg IV may be important in promoting prostate tumor growth during the transition from hormone dependent to hormone refractory status.

15. SUBJECT TERMS
Metastatic Prostate Cancer, Polyclonal Antibody, Sirna, Immunohistochemistry

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	11	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

New markers for aggressive and potentially metastatic prostate cancer are needed. To test the role of Reg IV, a secreted protein expressed in most metastatic prostate cancers, as a tissue and serum marker, we determined whether the expression of Reg IV in primary tumors correlates with the risk of recurring and developing progressive disease after surgery. Second, we determined the role of Reg IV in progression to androgen independence and/or metastasis. Finally, if we find a role for Reg IV in either of these processes, we will ask if neutralizing antibodies against Reg IV can be used therapeutically in animal models.

PROGRESS REPORT

Aim 1: *To determine if Reg IV expression in primary tumors correlates with risk of recurrence or PSA-doubling time after recurrence*

Task 1a: Polyclonal anti-Reg IV antibody production.

Initially, we developed the polyclonal rabbit antibody using the peptides proposed in our grants: aa 69 – 84, and aa 138 – 153. However, the titer of the harvested serum was too low, therefore the peptide regions were changed. Subsequently new synthetic peptides, RSWSGKSMGGNKHC (corresponding to residues 94–107), with a native C-terminal cysteine, and TIAEYISGYQRSQPC (residues 56–69), with a non-native C-terminal cysteine were synthesized, and coupled to maleimide-KLH. Anti-Reg IV polyclonal antibodies were produced by immunizing rabbit with the KLH-coupled synthetic peptides. Western blot analysis showed high titer of anti-RegIV antibody after third immunization (Figute 1). To affinity purify anti-RegIV polyclonal antibody, antigenic peptides were covalently attached to Sulfolink-coupling gel via cysteine residue in a column (Pierce, IL). Antisera were then run through the gel column, and the antibodies were eluted from the affinity column. Figure 2 showed that Reg IV protein can be detected specifically by affinity purified anti-RegIV antibodies.

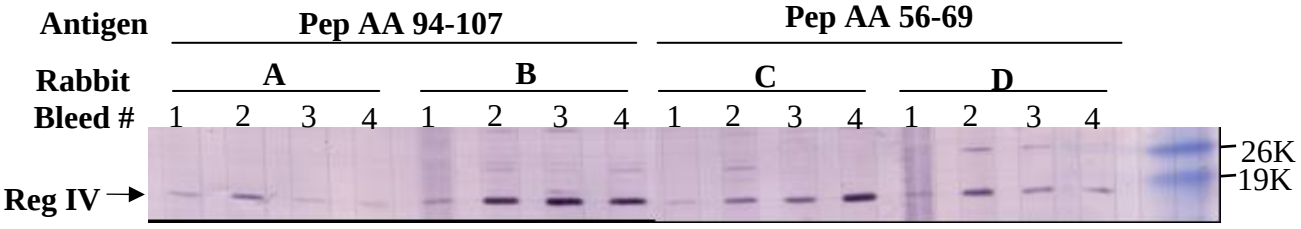


Figure 1. High titer of anti-Reg IV poyclonal antibody after third immunization.

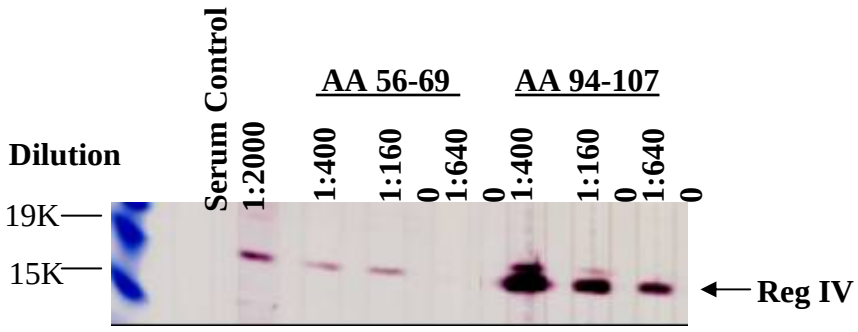


Figure 2. Detection of Reg IV protein by affinity purified antibody.

Task 1b: Development of monoclonal anti-Reg IV.

For the antigen, we had proposed to produce GST-Reg IV fusion protein, but subsequently encountered technical difficulties, and such production was unsuccessful. The GST-Reg IV fusion protein expressed in bacteria was insoluble due to the high contents of cysteine in the sequence. To circumvent this problem, Reg IV was expressed as a myc-His fusion protein in Drosophila S2 cells (Initrogen, CA). Using this system, we were able to express and detect the secreted Reg IV-myc-His fusion protein in S2 cell culture media (Figure 3). To develop the mouse monoclonal antibody, we used Reg IV-myc-his fusion protein to immunize Balb/c mice and measured their anti-Reg IV activity. The antibody titer was too low to proceed to the next step-fusion procedure. One alternative was to use the same KLH-peptides for making polyclonal antibody to immunize mice to make the monoclonal antibody. Due to lack of personnel, it was not feasible to concentrate on making another monocolonal antibody.

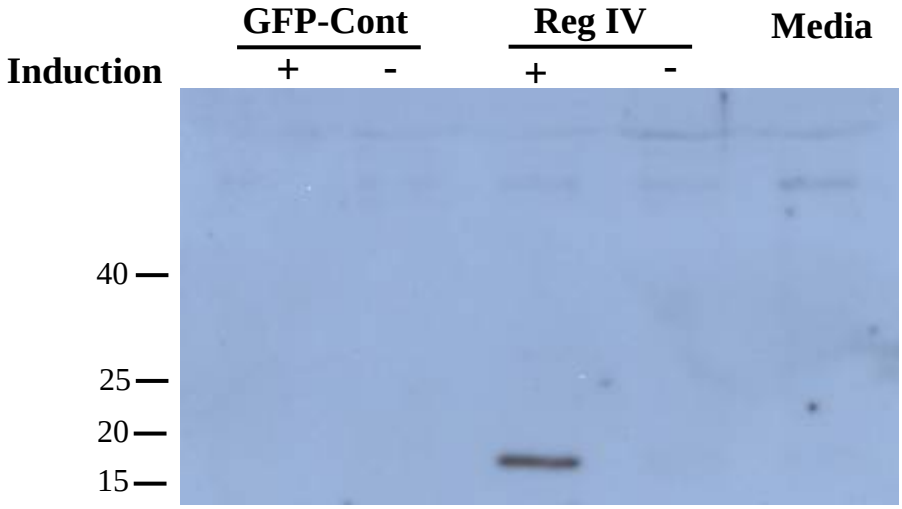


Figure 3. Expression of Recombinant Reg IV protein in Drosophila S2 cells.

Task 2: Construction of new tissue arrays

The new tissue array are being constructed by our collaborator.

Task 3: Immunohistochemistry

Using the affinity purified anti-Reg IV antibody, we were able to detect a strong signal in the xenograft tumors LAPC-9 AI (positive control), as opposed to no signal in LAPC-9 AD (negative control, figure 4). We also detected strong signal in a prostate cancer sample (figure 5). Further testing was being performed to address the nuclear staining that was observed in the cancer sample.

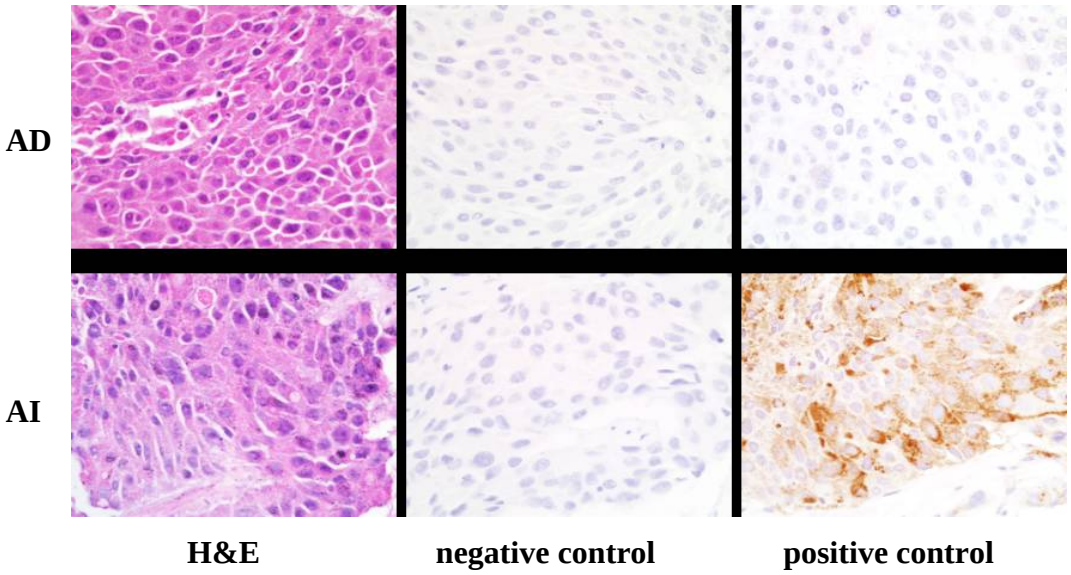


Figure 4. Detection of Reg IV protein in LAPC9-AI xenograft.

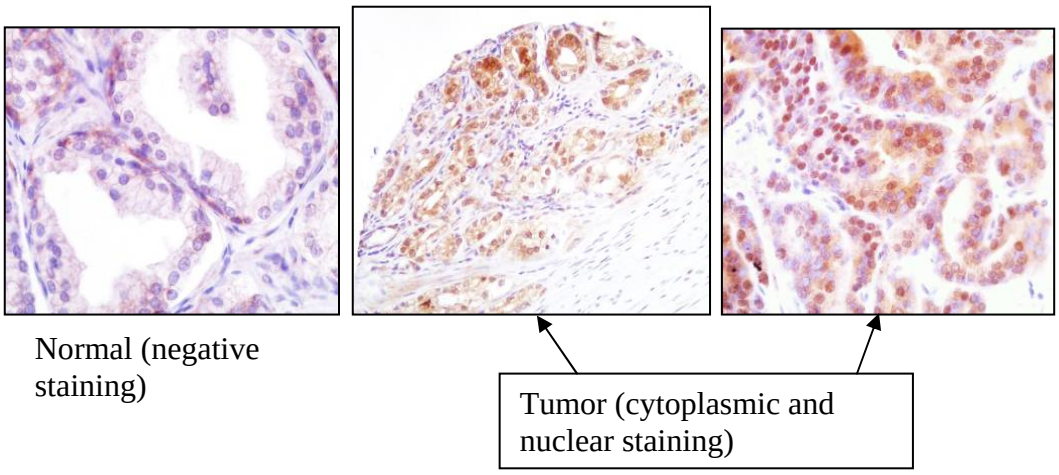


Figure 5. Detection of Reg IV protein in a human prostate cancer sample.

The antibody was tested in different prostate cancer specimens but due to problem of high background, more optimizations were required before actual staining can be performed on the tissue arrays.

Aim 2: To detect Reg IV by Enzyme-linked Immunoassay (ELISA) in sera of patients with primary and metastatic prostate cancer

Task 1: ELISA development

Affinity purified polyclonal anti-Reg IV was biotin labeled and antibody coating conditions were tested. When the plates were tested using the RegIV conditioned media, there was very high background.

Task 2: Clinical analysis, trouble shooting.

The development of ELISA was suspended due to difficulties in troubleshooting the problems, as well as a lack of personnel.

Aim 3: To determine if Reg IV plays a role in prostate cancer progression or resistance to therapy

Task 1: Overexpression of Reg IV.

LNCaP cells were stably transfected with pCDNA-REG IV expression vector or pCDNA control vector. The cells were grown on 96 well plates and measured for growth rate by MTT. The growth assay clearly showed that LNCaP-Reg IV cells grow faster than LNCaP control cells (figure 6). We also observed growth advantage in DU145-RegIV cells (transduced by CCR-RegIV lentivirus) over DU145 control cells (figure 7). However, no growth difference was observed between PC3-Reg IV and PC3 control.

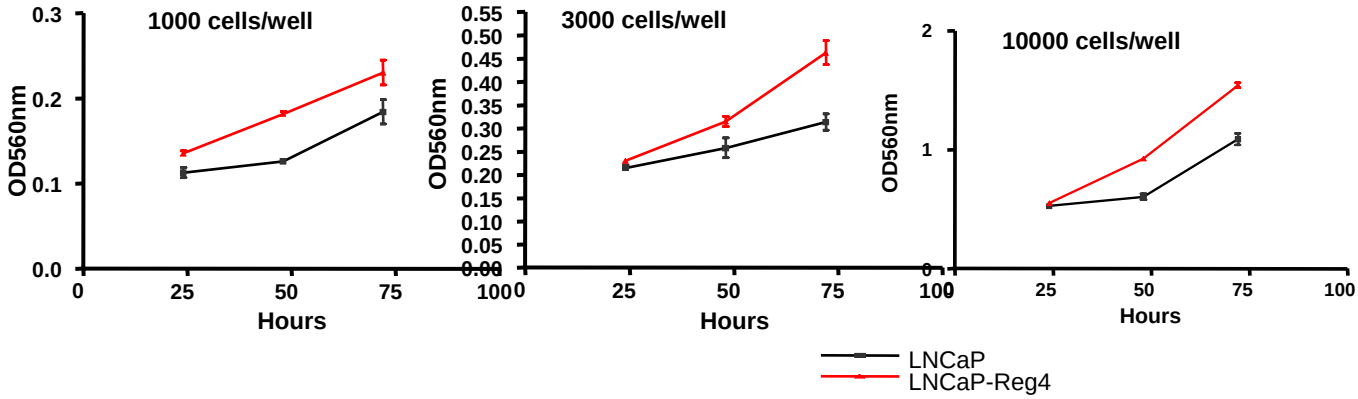


Figure 6. Reg IV-overexpressing LNCaP cells showed faster growth than control cells.

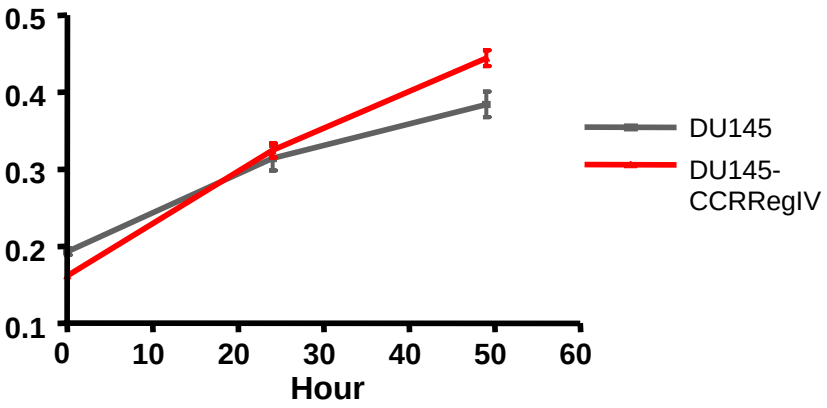


Figure 7. Reg IV promotes DU-145 cell growth in vitro

To test the effect of Reg IV on tumor cell growth in vivo, LAPC-9 cells were transduced with either Reg IV lentivirus or control virus and injected into immediately castrated mice or delayed castrated ones. As shown in figure 8, LAPC9-Reg IV appeared to have no growth advantage over control cells under both conditions.

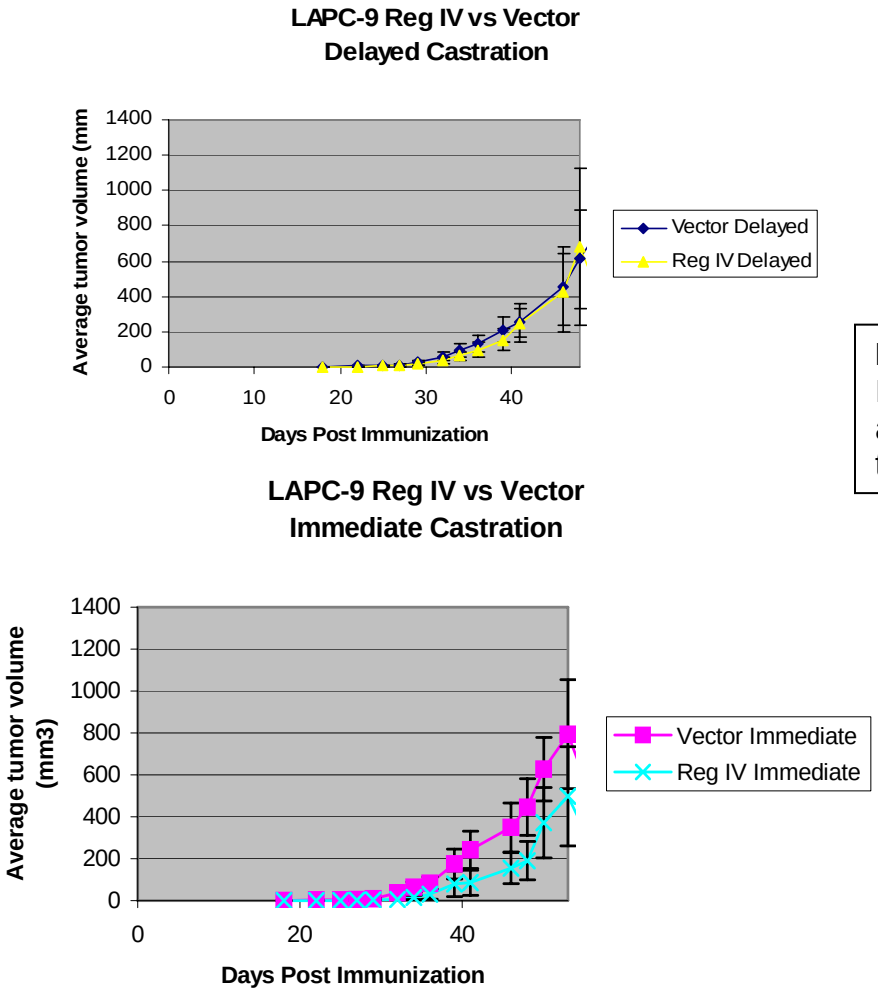
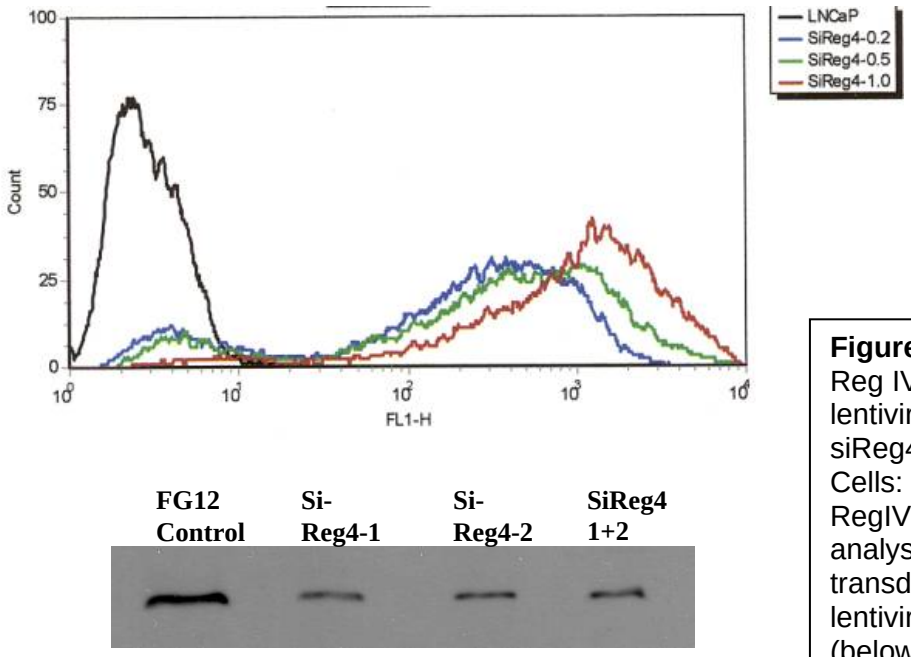


Figure 8. LAPC9-Reg IV had no growth advantage compare to LAPC9 in vivo.

Task 2: Knockdown of Reg IV.

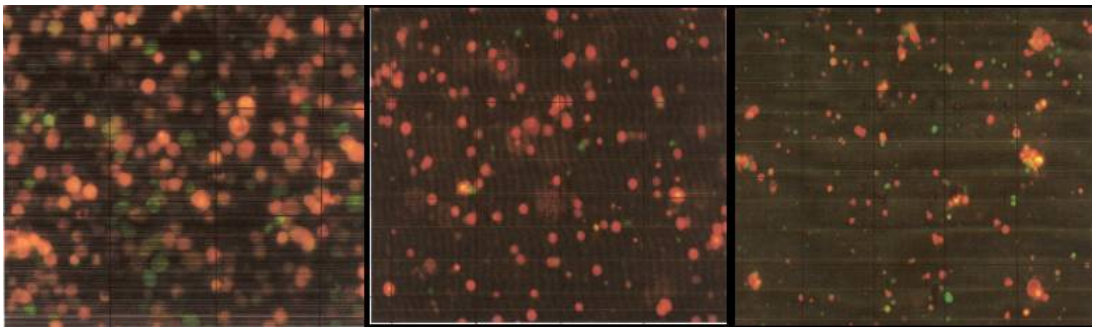
By collaboration with Irvin Chen's group, we designed siRNA lentiviral constructs against Reg-IV to knock down its gene and protein expression. We generated two siRNA constructs specifically targeting Reg-IV, but not other members of the Reg family. Both constructs successfully down-regulated Reg IV gene expression efficiently, as tested in LNCaP-pCDNA-Reg IV cells (figure 9).



SiReg 4-1: CTTCAGGAAGCTGAGGAAC
SiReg 4-2: AAGTGGCTATCAGAGAAGC.

Figure 9. Knockdown of Reg IV by GFP-expressing lentiviral constructs siReg4-1 and siReg4-2. Cells: LNCaP-PcDNA-RegIV.His.Myc. FACS analysis showed high transduction of the lentiviruses; Western blot (below) showed reduced expression of Reg IV compared to control.

The constructs was then used in an *in vivo* competition assay to analyze the effect of Reg IV gene expression in tumor growth. The assay was carried out using the LAPC9 xenograft tumor cell line. LAPC9 tumor cells, prepared as single cell suspension, were transduced with FG12siReg4-1 and –2 lentiviruses, which stably expressed GFP. For control, LAPC9 cells were transduced with CCR-DsRed lentivirus and verified to stably express the red fluorescent protein. We mixed together equal amount of LAPC9-DsRed (red) and LAPC9-SiReg-IV (green) cells, and inoculated the cells into intact (AD), as well as castrated (early castration, AI) nude mice. Alternatively, we first inoculated the cell mixture into intact mice, then performed castration once the tumors became palpable (AD – AI transition). Tumors were harvested for single cell suspensions and the number of red and GFP positive cells were counted. For both the AD and AI group, the ratio of red to green cells was 2:1, while in the case of delay castration, the ratio was 6:1 (figure 10). This indicated that control cells may have growth advantage over cells in which Reg IV was reduced. Thus Reg IV may promote LAPC9 xenograft tumor growth in transition from AD to AI status.



AD
Control (red) : SiRegIV (GFP)
2 : 1

AD → AI
Late castration
Control (red) : SiRegIV (GFP)
6 : 1

AI
Early castration
Control (red) : SiRegIV (GFP)
2 : 1

Figure 10. In vivo competition assay in LAPC9 cells for effect of knocking down Reg IV.

Task 3: antibody blockade in vivo.

Due to difficulties in producing high titer monoclonal antibody, the in vivo experiment on antibody blockade was not explored.

KEY RESEARCH ACCOMPLISHMENTS

- Affinity purify a polyclonal anti-RegIV antibody
- Able to express and detect the RegIV fusion protein in cell culture media
- Able to detect strong signal in immunohistochemistry control samples using the affinity purified polyclonal anti-RegIV antibody.
- Observed growth advantage in two prostate cancer lines transduced with RegIV.
- Down regulated RegIV gene expression using si-RegIV.
- In vivo experiment showed no growth advantage in LAPC9 transduced with Reg IV.
- In vivo RegIV knockdown experiment showed that Reg IV may promote LAPC9 xenograft tumor growth in transition from AD to AI status.

REPORTABLE OUTCOMES

None

CONCLUSION

We developed and purified a polyclonal anti-RegIV that was successfully been tested by immunohistochemistry on positive and negative controls; however, nuclear staining was observed in the prostate cancer sample. Once the nuclear staining background has been worked out on the cancer samples, the anti-RegIV polyclonal could be used to stain the prostate tissue array. We encountered difficulties in developing a monoclonal anti-RegIV antibody due to the insolubility of the fusion protein and its high cysteine content. Using an alternative system in Drosophila, the fusion protein could be expressed and detected, but the antibody titer was too low to proceed to the next step-fusion procedure. *In vitro* studies show cells overexpressing RegIV have a growth advantage, however in vivo there was no clear difference. Using siRNA tecknology to knock down RegIV RNA in an *in vivo* competition assay, we observed that control cells have better growth advantage than knockdown cells upon androgen withdrawal. Thus Reg IV may promote LAPC9 xenograft tumor growth in transition from AD to AI status.

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